FoxO3a Is an Antimelanogenic Factor that Mediates Antioxidant-Induced Depigmentation

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Forkhead box-O (FoxO) family transcriptional factors control the expression of many genes involved in a variety of cellular processes. Melanogenesis is an oxidizing process; therefore, many antioxidants are used to inhibit melanin production. However, their mechanism of action is poorly understood. In this study, we investigated the role of FoxO3a, which is a key factor in oxidative stress–related cellular responses in melanogenesis. When FoxO3a expression was inhibited, the expression of melanogenic genes and melanin levels increased. In contrast, the overexpression of wild-type FoxO3a and the increased nuclear translocation induced by the phosphoinositide 3-kinase inhibitors or by Akt inhibition reversed these phenomena. This effect was not observed when FoxO3a harbored a deletion in the nuclear localization signal, indicating that its nuclear translocation is important for the regulation of melanogenesis. When antioxidants such as vitamin C, *N*-acetylcysteine, and Trolox were applied to MNT1 cells, melanin levels decreased in parallel with FoxO3a nuclear translocation, and this effect disappeared with FoxO3a-directed small interfering RNA treatment. Because FoxO3a orchestrates the expression of many genes in order to regulate cellular phenotypes in a variety of environmental states, this gene, a factor involved in melanogenesis regulation, may represent a good target for studying antimelanogenic signaling pathways and for designing pharmacological or antimelanogenic agents that regulate melanin synthesis.

Journal of Investigative Dermatology (2014) **134,** 1378–1388; doi:10.1038/jid.2013.510; published online 2 January 2014

INTRODUCTION

Class O forkhead/winged helix transcription factors (FoxO), which are widely conserved from *Caenorhabditis elegans* to mammals, display a wide range of organismal functions that orchestrate gene expression programs activated by a variety of cellular processes, such as the cell cycle, apoptosis, DNA repair, energy metabolism, development, and protection from oxidative stress (Nakae *et al.*, 2002; Blucher *et al.*, 2003; Holzenberger *et al.*, 2003; Hu *et al.*, 2004; Paik *et al.*, 2007). Four members of the mammalian FoxO family have been identified: FoxO1, FoxO3a, FoxO4, and FoxO6. These FoxO transcription factors are involved in cellular signaling and are triggered by a variety of environmental stimuli, such as insulin, IGFs, oxidative stress, cytokines, and nutrients. In addition, they may be critical to preventing aging and age-dependent diseases. All FoxO proteins bind to the same consensus sequence and hence can act in a redundant manner. However, specific functions for particular FoxO isoforms have been described, and this specificity can be partially ascribed to their tissue-specific expression patterns (Biggs *et al.*, 2001; Jacobs *et al.*, 2003).

Deletions of individual *FoxO* genes in mice have revealed redundant isoform-specific functions of FoxO1, FoxO3a, and FoxO4 (Van der Vos *et al.*, 2011a, 2011b). The deletion of FoxO1 is lethal as a result of incomplete vascular development (Hosaka *et al.*, 2004). FoxO3a-knockout mice show age-dependent infertility as a result of global ovarian follicle activation, which results in early oocyte depletion (Castrillon *et al.*, 2003). In contrast, no phenotype has yet been observed for FoxO4 depletion (Hosaka *et al.*, 2004). These results suggest that the *FoxO* genes have critical roles in a wide variety of cellular processes, particularly FoxO3a, which functions in age-dependent cellular processes.

Each FoxO family is exquisitely regulated by posttranslational modifications, which affect protein activity, cellular localization, and stability. Reactive oxygen species (ROS) regulate FoxO activity either by modulating the activity of growth factor receptors, tyrosine kinases, serine/threonine kinases, and phosphatases or by regulating lipid metabolism through interactions with factors that are upstream of FoxO (Van der Heide *et al.*, 2004; Obsil and Obsilova, 2008; Patridge and Brüning, 2008). ROS inhibit or activate FoxO factors in a context-dependent manner. Hydrogen peroxide inhibits FoxO activity by activating the phosphoinositide

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Abbreviations: cKIT, SCF receptor; DCT, DopaChrome Tautomerase; FoxO, forkhead box-O; MART1, melanoma antigen recognized by T cells; MITF, microphthalmia-associated transcription factor; NAC, N-acetylcysteine; PAX3, paired box transcription factor; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RNAi, RNA interference; ROS, reactive oxygen species; RT-qPCR, quantitative real-time reverse-transcriptase–PCR; SCF, stem cell factor; siRNA, small interfering RNA; SOX10, SRY-related HMG-box; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1; Vc, vitamin C; WT, wild type

Received 10 July 2013; revised 24 October 2013; accepted 6 November 2013; accepted article preview online 27 November 2013; published online 2 January 2014

3-kinase (PI3K)-protein kinase B pathway by exerting insulin-mimetic effects (Heffetz *et al.*, 1992), whereas ROS–activated macrophage-stimulating 1, mitogen-activated protein kinase, and c-Jun N-terminal kinase activate the FoxO proteins via phosphorylation and disruption of binding to the 14-3-3 protein (Ueda *et al.*, 2002; Essers and Weijzen, 2004; Bi *et al.*, 2010). These studies suggest that FoxO activity can be positively or negatively regulated depending on the degree of ROS accumulation and the duration of ROS signaling.

In skin, downregulation of FoxO3a accelerates cellular senescence in human dermal fibroblasts (Kim *et al.*, 2005). As a pro-apoptotic gene, *FoxO3a* is negatively regulated by Notch signaling in UVB–treated keratinocytes, thereby resulting in protection against UVB radiation (Mandinova *et al.*, 2008), and its nuclear translocation is also regulated by UV irradiation through the c-Jun N-terminal kinase-extracellular signal-regulated kinase/AKT pathway, which promotes apoptosis (Wang *et al.*, 2012). These studies suggest that FoxO3a has an important role in cellular responses against external stimuli in the skin, but the function of FoxO3a in melanocytic cells has not yet been elucidated. As a photoprotector for skin against UV irradiation, melanin is produced by sequential oxidation processes through which tyrosine is converted to eumelanin and pheomelanin. Because of their oxidative properties, many antioxidants, such as vitamins C and E, have been used to treat skin hyperpigmentation (Lerner and Fitzpatrick, 1950; Hayakawa, 1980). In addition, the low activity of catalase, the main enzyme responsible for degrading hydrogen peroxide, is associated with the low skin phototype; patients with this phototype burn relatively easily, do not tan well, and are at a higher risk for skin cancer compared with those with the high skin phototype (Picardo *et al.*, 1999), thereby suggesting a close relationship between melanogenesis and redox state in the skin. However, the mechanism that regulates redox state–dependent melanogenesis has not yet been studied in detail. Because FoxO3a activity can be regulated by ROS, which in turn regulates melanogenesis, we speculate that the melanogenesis alteration induced by ROS perturbation may occur via FoxO3a-mediated cell signaling.

In this study, we show that FoxO3a is an antimelanogenic factor. We show that the knockdown of FoxO3a expression using small interfering RNAs (siRNAs) greatly induced melanin production and increased the expression of proteins that are associated with melanogenesis. In contrast, the overexpression of FoxO3a and the inhibition of PI3K or Akt resulted in increased FoxO3a nuclear translocation and consequently decreased melanogenesis. Furthermore, we verified the involvement of FoxO3a in antioxidant-mediated depigmentation, showing that antioxidants promoted the nuclear translocation of FoxO3a and that FoxO3a-directed siRNA diminished these antioxidant effects.

RESULTS

FoxO3a positively regulates melanogenesis in pigmented cells

FoxO3a has an important role in various pathways associated with cell type– and environment-specific differentiation by regulating the cell cycle machinery (Hu *et al.*, 2008). In skin cells, Foxo3a has a role both in the senescence of dermal fibroblasts and in the response of keratinocytes to UV irradiation. To investigate whether *FoxO* genes are involved in melanocyte differentiation, we first assessed the expression levels of FoxO1, FoxO3a, and FoxO4 in lightly pigmented (LP), moderately pigmented (MP), and darkly pigmented melanocytes and melanoma MNT1 cells (Figure 1a–c). Of the FoxO family members, FoxO3a was expressed at more than 10-fold higher levels than other FoxO proteins in these pigment-related cells (Figure 1b), and its expression levels decreased with increasing degree of pigmentation (Figure 1b and c), suggesting an inverse relationship between FoxO3a expression and melanin levels. To confirm that FoxO3a is involved in melanogenesis, we treated cells with the PI3K inhibitors LY294002 and wortmannin, which induce FoxO3a activation (Yao and Cooper, 1995). When PI3K inhibitors were applied to primary melanocytes, FoxO3a significantly translocated to the nucleus (Figure 1d), melanin levels decreased (Figure 1e), and the pigment-related proteins tyrosinase (TYR), TYR-related protein 1 (TYRP1), and dopachrome tautomerase (dopachrome delta-isomerase, TYR-related protein 2, DCT) were downregulated (Figure 1f).

To verify that Foxo3a is the main factor in melanogenesis regulation, we introduced siRNAs against FoxO3a into human melanoma MNT1 cells, which are highly pigmented melanotic cells with mature stage III and IV melanosomes and express relatively high levels of melanin (Figure 1a). We used two different types of FoxO3a-directed siRNAs (si-1 and si-2) and verified that both were functioning properly by qPCR and immunoblot assay (Figure 2a and c). When FoxO3a-directed siRNAs were introduced into cells, the expression of stem cell factor (SCF), the SCF receptor (cKIT), and SRY-related HMG-box (SOX10), which are associated with proliferation rather than melanogenesis, was downregulated, whereas the expression of pigment-related genes, such as microphthalmia-associated transcription factor (MITF), TYR, TYRP1, and DCT, was upregulated (Figure 2a and b). We further examined the expression of several genes that have a role in early to late melanogenesis. Compared with the BRN2, S100β, and NOTCH1 levels, which were downregulated, the mRNA levels of paired box transcription factor (PAX3), melanoma antigen recognized by T cells (MART1), endothelin receptor B (EDNRB), and p75NTR were increased by siRNAs against FoxO3a (Supplementary Figure S1a and b online). Consistent with the upregulation of pigment-related genes, the protein levels of MITF, TYR, TYRP1, and DCT accumulated (Figure 2c). Moreover, melanin levels (Figure 2d and e) and TYR activity (Figure 2f) were significantly increased in FoxO3a-directed siRNA-treated cells compared with control or mock (negative control siRNA)-treated cells, suggesting that FoxO3a inhibits melanogenesis by downregulating pigment-related genes.

Elevated nuclear FoxO3a inhibits melanogenesis

FoxO family proteins function as downstream effectors of the PI3K-protein kinase B (also called AKT) pathway (Burgering and Kops, 2002). At the molecular level, activated PI3K-protein kinase B phosphorylates FoxO3a, which reduces

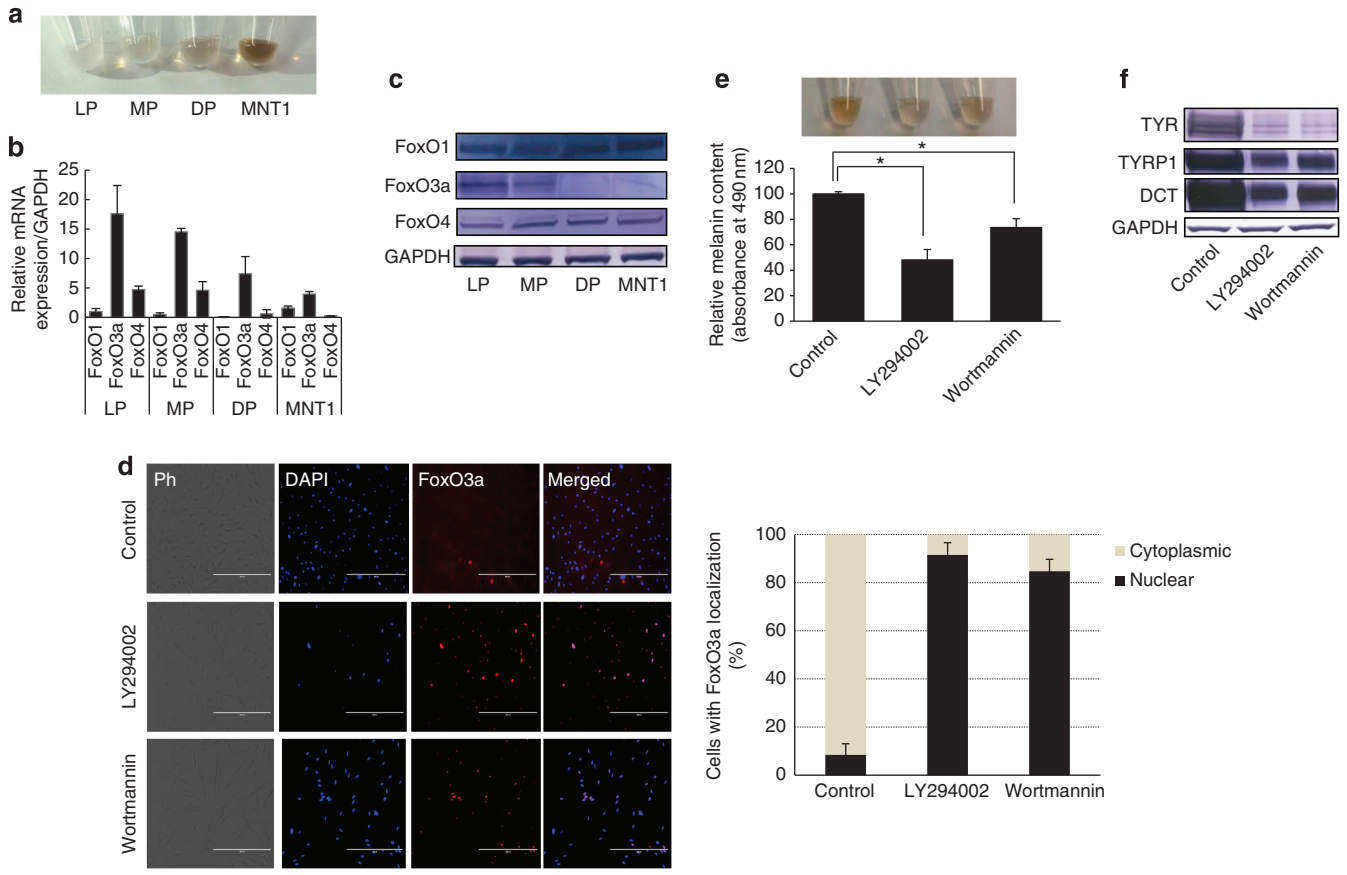

Figure 1. The expression of forkhead box-O (FoxO) proteins in pigmented cells and the regulation of melanogenesis by FoxO3a in primary melanocytes. (**a**) The melanin levels in lightly pigmented (LP), moderately pigmented (MP), and darkly pigmented (DP) melanocytes and MNT1 cells were visualized after dissolving cell pellets in 1 N NaOH. (**b**) The mRNA expression levels of FoxO1, FoxO3a, and FoxO4 in the LP, MP, and DP melanocytes and in MNT1 cells. The data represent three independent experiments (mean ± SD). (**c**) The protein expression levels of FoxO proteins in pigmented cells were determined by western blot analysis. (**d**) Primary melanocytes were treated with the phosphoinositide 3-kinase (PI3K) inhibitors LY294002 (500 μM) or wortmannin (1 μM) for 48 hours. The cells were stained with an anti-FoxO3a antibody (FoxO3a; red) and 4′,6-diamidino-2-phenylindole (DAPI) (nucleus; blue). Images were obtained by fluorescence microscopy and merged. Representative images are shown. Control, vehicle alone treated; Ph, phase-contrast image. Bar = 200 μm. Cells with FoxO3a localization were counted and depicted by means of an EVOSfl digital fluorescence microscope. (**e**) The PI3K inhibitors LY294002 (500 μM) and wortmannin (1 μM) were used to treat primary melanocytes for 5 days, and the melanin levels were then visualized. The quantitative melanin levels were determined by measuring absorbance at 490 nm using a spectrophotometer. *$P < 0.05$. (**f**) Primary melanocytes were treated with the PI3K inhibitors LY294002 (500 μM) or wortmannin (1 μM) for 48 hours, and then the cell lysates were analyzed by western blotting. Control: vehicle alone treated.

FoxO3a nuclear translocation and suppresses its transactivation activity (Brunet *et al.*, 1999; Kops *et al.*, 2002). Therefore, we used the well-known PI3K inhibitors LY294002 and wortmannin to induce FoxO3a nuclear translocation and activation (Yao and Cooper, 1995). When PI3K inhibitors were applied to cells, the melanin levels decreased by 59% (LY294002) and 60.3% (wortmannin) compared with those in untreated groups (Figure 3a and b), and the TYR activity was reduced by 57.8% and 54.6%, respectively (Figure 3c). The mRNA expression levels of the melanogenesis-related genes *MITF*, *TYR*, *TYRP1*, *DCT*, *PAX3*, and *EDNRB* were reduced (Figure 3d and Supplementary Figure S1c online), whereas the early-stage markers of melanogenesis, such as SCF, cKIT, SOX10, BRN2, and NOTCH1, were increased, which is in contrast to the results shown for FoxO3a-directed siRNA expression (Figure 3e and Supplementary Figure S1d online). We found that PI3K inhibitors increased the mRNA and protein expression levels of FoxO3a and subsequently enriched the protein in the nuclear fraction (Figure 3e and f). In accordance with the accumulation of FoxO3a in the nucleus, the protein expression of *MITF*, *TYR*, *TYRP1*, and *DCT* was downregulated, suggesting that the elevated levels of FoxO3a in the nuclear fraction inhibit melanogenesis (Figure 3f).

Nuclear translocation of FoxO3a is a key process in melanogenesis regulation

As shown using FoxO3a-directed siRNA and PI3K inhibitors, the levels and nuclear translocation of FoxO3a are associated with melanogenesis; however, whether the level or location of FoxO3a is more closely associated with melanogenesis regulation remains unclear. To examine the effect of FoxO3a nuclear translocation on melanogenesis, we constructed plasmids expressing wild-type (WT) FoxO3a (plasmid 1787: HA-FoxO3a; WT) or mutant FoxO3a with a deletion in the nuclear localization sequence (NLS), an essential region for

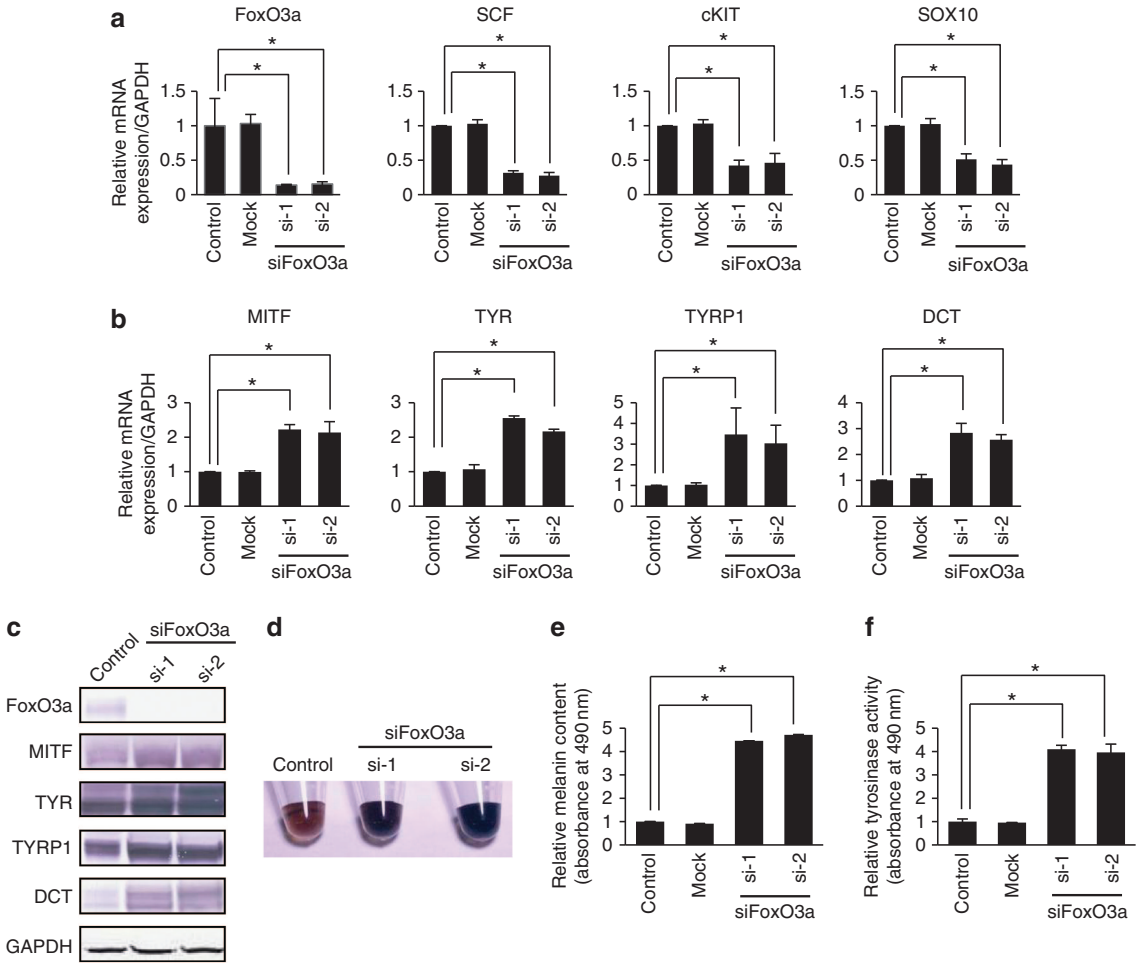

Figure 2. Forkhead box-O3a (FoxO3a) regulates melanogenesis by modulating melanogenic genes. MNT1 cells were transfected with small interfering RNAs (siRNAs) against FoxO3a for 48 hours. Genes that were (**a**) downregulated or (**b**) upregulated were analyzed by means of quantitative real-time reverse-transcriptase–PCR (RT-qPCR). *$P<0.05$. (**c**) After treatment with FoxO3a siRNAs, the cell lysates were analyzed by western blot analysis. The (**d**, **e**) melanin levels and (**e**, **f**) tyrosinase activity were assessed by measuring absorbance at 490 nm with a spectrophotometer. Control: untreated; mock: control siRNA; si-1, -2: siRNA against FoxO3a. The values are presented as the mean ± SD of three independent experiments. *$P<0.05$. cKIT, DCT, DopaChrome Tautomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MITF, microphthalmia-associated transcription factor; SCF, stem cell factor; SOX10, SRY-related HMG-box; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1.

FoxO3a function (Brownawell *et al.*, 2001) (plasmid 8361: FLAG-FoxO3a TM; ΔNLS). When MNT1 cells were transfected with the WT or mutant constructs, the total level of FoxO3a proteins greatly increased under both conditions (Figure 3g, lower panel, whole FoxO3a), whereas the nuclear FoxO3a levels were increased only under the WT overexpression condition (Figure 3g, upper panel). In addition, the melanin levels greatly decreased in the WT–expressing cells but not in the mutant-expressing cells (Figure 3h), indicating that nuclear translocation, rather than Foxo3a protein levels, is associated with melanogenesis. FoxO3a activation is induced by its nuclear translocation (Van der Heide *et al.*, 2004). Therefore, the regulation of melanogenesis by FoxO3a is dependent on its activation through nuclear translocation. To confirm that FoxO3a nuclear translocation is the main factor for melanogenesis inhibition, we next assessed Akt inhibition by Akt-directed siRNA or an Akt inhibitor. The treatment with siRNA against Akt or with an Akt inhibitor decreased melanin by 61.7% and 59%, respectively, whereas FoxO3a-directed siRNA increased melanin production by 114.8% (Figure 3i). The phosphorylation of FoxO3a was blocked by the inhibition of Akt (Figure 3j), and the nuclear translocation of FoxO3a was greatly increased by Akt inhibition (Figure 3k). On the basis of these results, we suggest that FoxO3a nuclear translocation by either PI3K or Akt inhibition results in the suppression of melanogenesis.

FoxO3a mediates the antimelanogenic activity of antioxidants and PI3K inhibitors

Because several enzymatic oxidation steps occur during melanin synthesis, many antioxidants have been used to treat skin hyperpigmentation. Hydroquinone, vitamin C (Vc), vitamin E, and N-acetylcysteine (NAC) have been widely used to reduce melanin levels, and their synergetic effects have been shown (Fujiwara *et al.*, 2004; Hu *et al.*, 2009). However, the mechanism by which antioxidants regulate melanogenesis has

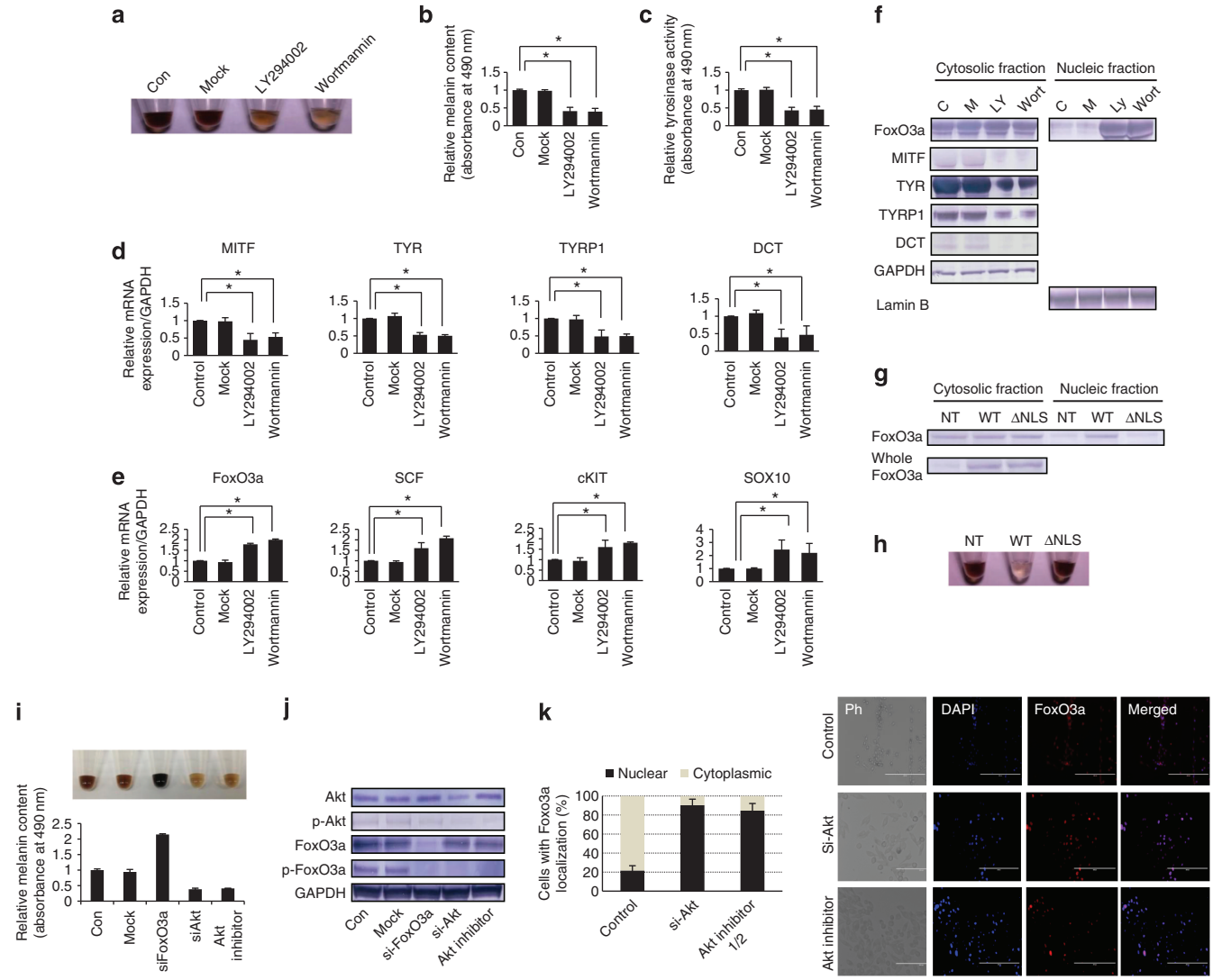

Figure 3. Melanogenesis regulation by forkhead box-O3a (FoxO3a) is mediated via its nuclear translocation. (**a**) MNT1 cells were treated with the phosphoinositide 3-kinase (PI3K) inhibitors LY294002 (500 μM) and wortmannin (1 μM) for 48 hours, and the melanin levels were visualized. The (**b**) quantitative melanin levels and (**c**) tyrosinase activity were determined by measuring absorbance at 490 nm using a spectrophotometer. After treatment with the PI3K inhibitors, the expression of various genes was examined by quantitative real-time reverse-transcriptase–PCR (RT-qPCR), and the (**d**) downregulated and (**e**) upregulated genes are shown. $*P<0.05$. The data are presented as the mean ± SD of three independent experiments. (**f**) The cells were fractionated into cytosolic and nuclear portions after PI3K inhibitor treatment, and each protein, including FoxO3a, was analyzed by western blot analysis. Lamin B was used as a marker of the nuclear fraction. C, untreated; M, vehicle alone treated; Ly, LY294002; Wort, wortmannin. (**g**) Constructs for wild-type FoxO3a (WT) or mutant FoxO3a harboring a deleted nuclear localization signal (ΔNLS) were transfected into MNT1 cells, and the cells were fractionated into cytosolic and nuclear portions. FoxO3a expression was analyzed by western blot. NT, non-treated; ΔNLS, nuclear localization signal deleted. (**h**) The melanin levels were visualized after overexpression of the WT or mutant FoxO3a. The MNT1 cells were transfected with small interfering RNAs (siRNAs) against FoxO3a or Akt; the treatment with 10 μM Akt inhibitor was performed for 48 hours. (**i**) The melanin levels were visualized and quantified by measuring absorbance at 490 nm, and (**j**) the protein levels of Akt, phosphorylated (p)-Akt, FoxO3a, and p-FoxO3a were analyzed by western blot. Con: vehicle alone treated; mock: control siRNA. (**k**) MNT1 cells were transfected with siRNAs against FoxO3a or Akt; treatment with 10 μM Akt inhibitor was performed for 48 hours. The cells were stained with an anti-FoxO3a antibody (FoxO3a; red) and 4′,6-diamidino-2-phenylindole (DAPI) (nucleus; blue). The representative images were obtained under fluorescence microscopy and merged. Ph, phase-contrast image. Bar = 200 μm. Cells with FoxO3a localization were counted and depicted by means of an EVOSfl digital fluorescence microscope. cKIT, SCF receptor; DCT, DopaChrome Tautomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MITF, microphthalmia-associated transcription factor; SCF, stem cell factor; SOX10, SRY-related HMG-box; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1.

not yet been investigated in detail. To elucidate this mechanism, we first examined the effects of representative antioxidants, including Vc, NAC, and Trolox (a water-soluble derivative of vitamin E), on pigmentation. As previously reported (Fujiwara *et al.*, 2004), the melanin levels and TYR activity gradually decreased to a certain extent following antioxidant treatment. However, these effects were transient, and the melanin levels and TYR activity gradually increased with increases in antioxidant concentrations higher than 100 nM (Figure 4a). Although each antioxidant is structurally

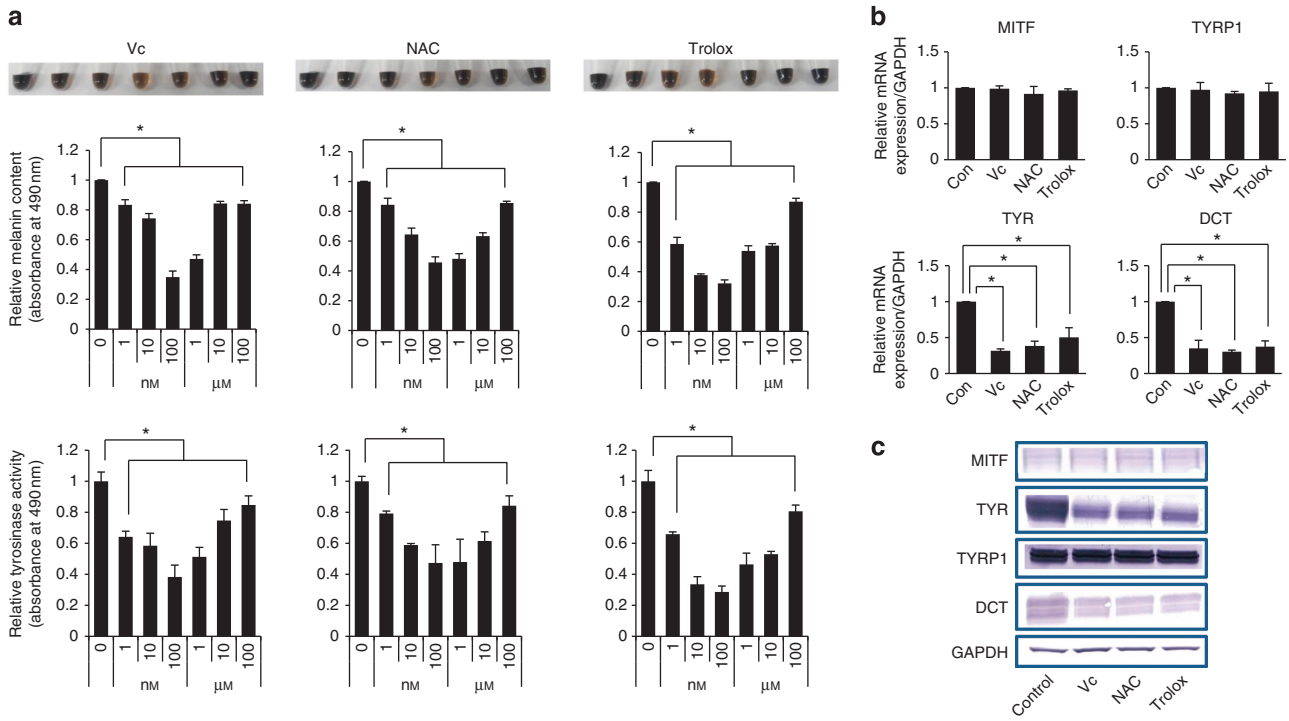

Figure 4. Antioxidants reduce melanogenesis by the inhibition of tyrosinase (TYR) and DopaChrome Tautomerase (DCT). (**a**) The melanin levels were visualized and quantified after treatment with each antioxidant at various concentrations for 48 hours by measuring absorbance at 490 nm (upper panel). The TYR activity is shown (lower panel). After treatment with each antioxidant (100 nM) for 48 hours, the mRNA and protein expression levels of melanogenesis-related genes were analyzed by (**b**) quantitative real-time reverse-transcriptase–PCR (RT-qPCR) and (**c**) western blot. *$P<0.05$. Con, vehicle only treated; Vc, vitamin C; NAC, *N*-acetylcysteine. The data shown are the mean ± SD of three independent experiments. cKIT, SCF receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MITF, microphthalmia-associated transcription factor; SCF, stem cell factor; TYRP1, tyrosinase-related protein 1.

different, they displayed very similar melanogenesis inhibition patterns. In the overall concentration range, *in vitro* TYR activity, cell proliferation, and cytotoxicity were not affected by each antioxidant (Supplementary Figure S2 online). Next, we examined the expression of pigment-related genes after treatment with 100 nM of each antioxidant. The mRNA and protein levels of MITF and TYRP1 remained unchanged, whereas the levels of TYR and DCT were significantly reduced (Figure 4b and c).

On the basis of a previous study showing the translocation of DAF-16 (a homolog of Foxo3a) from the cytosol to the nucleus in response to antioxidants in *C. elegans* (Kampkötter *et al*, 2007), we hypothesized that FoxO3a activation by nuclear translocation is involved in the antimelanogenic processes induced by antioxidants. To test this hypothesis, we performed immunofluorescence assays. Increasing concentrations of each antioxidant (Vc, NAC, and Trolox) induced the nuclear translocation of FoxO3a, showing the gradual enrichment of FoxO3a within the nucleus up to 100 nM and a gradual reduction at greater concentrations (Supplementary Figure S3 online). These results suggest that melanogenesis is modulated by Foxo3a nuclear translocation, which is in turn regulated by antioxidants. We confirmed the nuclear localization of FoxO3a after treatment with 100 nM of each antioxidant, which was the concentration that yielded the greatest antimelanogenic activity (Figure 5a). Without a change in the total protein levels (Figure 5b), endogenous FoxO3a was enriched in the nuclear fraction of antioxidant-treated cells compared with non-treated control cells (Figure 5c). To uncover how FoxO3a nuclear translocation occurs, we performed a time-course experiment. The results showed that FoxO3a localized to the nucleus after 2 hours and that this process gradually strengthened with time (Figure 5d). FoxO3a nuclear localization occurs relatively quickly and directly, and this response is continuous rather than transient (Figure 5d). Meanwhile, the nuclear translocation of another FoxO protein, FoxO1, induced by antioxidants was not observed (Supplementary Figure S4 online).

To ensure that the antimelanogenic effect of antioxidants was mediated by FoxO3a activation, we pretreated MNT1 cells with FoxO3a siRNA and then applied 100 nM of each antioxidant. As expected, cells that were pretreated with FoxO3a siRNA displayed increased melanin levels and TYR activity compared with control or mock-treated cells (Figure 6a-c). The increased melanin levels and TYR activity following the FoxO3a siRNA treatment were not significantly reversed by any antioxidant treatment (Figure 6a-c) at any dose (Figure 6d). In addition, the increased melanogenesis induced by FoxO3a siRNA was not reduced remarkably by treatment with the PI3K inhibitor (Figure 6e). Although some doses of antioxidants and wortmannin decreased the melanin in FoxO3a siRNA-treated samples, such decreases did not

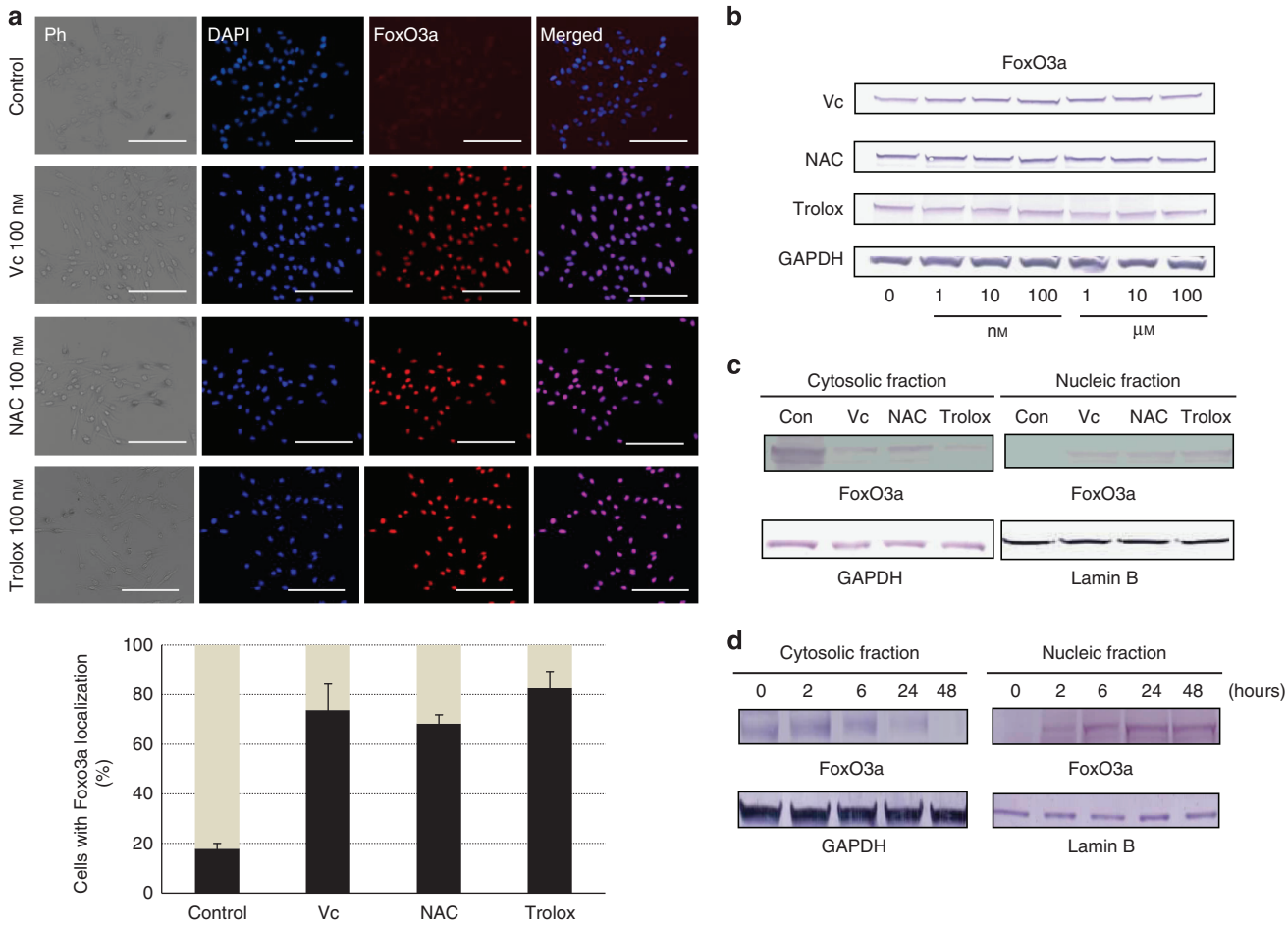

Figure 5. Antioxidants induce the nuclear translocation of forkhead box-O3a (FoxO3a). (**a**) The indicated antioxidants (100 nM) were added to MNT1 cells for 48 hours. The cells were stained with an anti-FoxO3a antibody (FoxO3a; red) and 4′,6-diamidino-2-phenylindole (DAPI) (nucleus; blue). The representative images were obtained under fluorescence microscopy and merged. Ph, phase-contrast image. Bar = 100 μm. Cells with FoxO3a localization were counted and depicted by means of an EVOSfl digital fluorescence microscope. (**b**) Total FoxO3a protein was analyzed by western blot analysis after treatment with each antioxidant at various concentrations. (**c**) MNT1 cells were treated with 100 nM of each antioxidant for 48 hours, and FoxO3a was analyzed by western blot after cellular fractionation. (**d**) The cells were treated with 100 nM of vitamin C (Vc) for 2, 6, 24, or 48 hours and then fractionated into cytosolic and nuclear portions. FoxO3a was detected by western blot. Con, vehicle only treated; NAC, *N*-acetylcysteine. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a marker of the cytosolic fraction and lamin B as a marker of the nuclear fraction.

occur to a distinct degree. These results imply that FoxO3a is an essential factor for the antimelanogenic activity of antioxidants and PI3K inhibitors.

DISCUSSION

Melanogenesis involves a series of tightly regulated enzymatic oxidation processes, which culminate in the synthesis and assembly of the melanin polymer. Therefore, many antioxidative agents have been used to treat skin hyperpigmentation; however, the regulatory mechanism is unknown. FoxO3a regulates many target genes that are involved in the antioxidative response in various cell types, including skin cells. In this study, we suggest that FoxO3a is involved in the regulation of melanogenesis and is a mediator of antimelanogenic effects. We verified that FoxO3a is an antimelanogenic factor by evaluating the effects of its knockdown and overexpression and the activation of FoxO3a using PI3K inhibitors and Akt inhibition. We also showed that the antimelanogenic activity of antioxidants is mediated by FoxO3a activation via its nuclear translocation.

The antimelanogenic effects of antioxidants and PI3K inhibitors are mainly mediated by FoxO3a activation

One possible explanation for the antimelanogenic effect of antioxidants is that they inhibit melanin synthesis by simply reducing cellular ROS levels. However, based on our results, the antimelanogenic effect of antioxidants appears to be mediated by FoxO3a activation (Figures 5 and 6), providing a reasonable molecular mechanism. In response to simple antioxidant treatments at various concentrations, we observed that the melanin levels and TYR activity were decreased to a certain extent; the maximum decrease was observed at 100 nM, and higher concentrations led to reduced effects. These phenomena were well coordinated with the nuclear

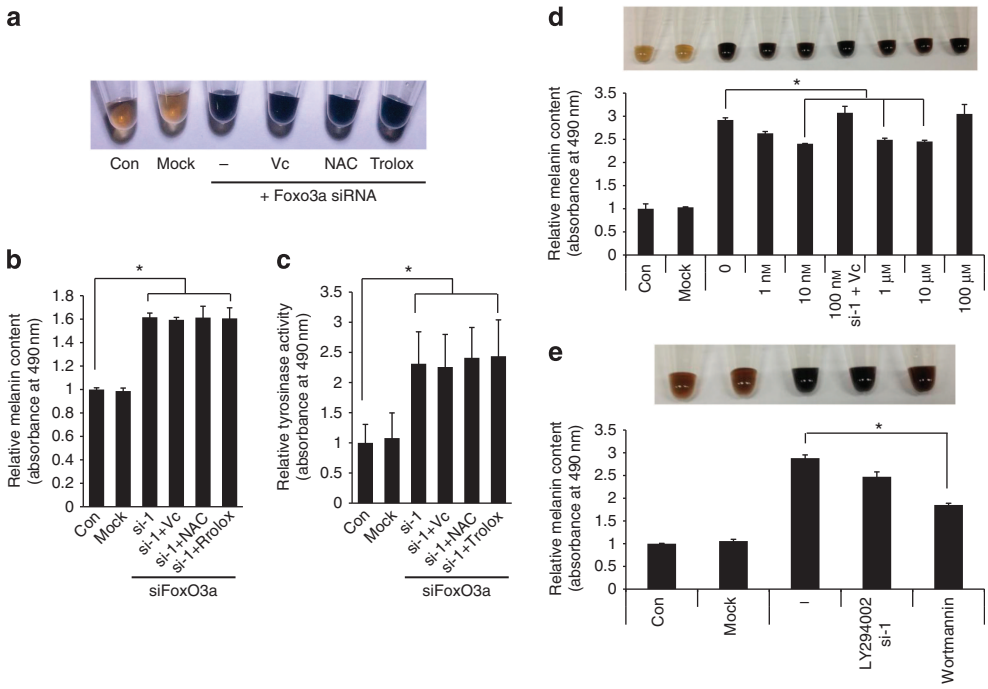

Figure 6. Forkhead box-O3a (FoxO3a) is a major target of melanin synthesis by antioxidant and phosphoinositide 3-kinase (PI3K). FoxO3a small interfering RNAs (siRNAs) were applied to MNT1 cells, and the cells were then treated with (**a–c**) 100 nM of each antioxidant, (**d**) a 0–100 μM dose range of vitamin C, or (**e**) with PI3K inhibitors. (**a**, **b**, **d**, **e**) The melanin levels were visualized and quantified, and (**c**) tyrosinase activity was determined by measuring absorbance at 490 nm. Con, vehicle alone treated; mock, control siRNA treated; si, siRNA against FoxO3a; Vc, vitamin C; NAC, *N*-acetylcysteine. The data shown are the mean ± SD of three independent experiments. *$P<0.05$.

translocation of FoxO3a (Figures 4 and 5 and Supplementary Figure S3 online). Hermetic (bell-shaped or U-shaped) concentration effect curves are often observed for many chemicals, including toxins, drugs, oxidants, and antioxidants (Cornelius *et al.*, 2013). FoxO transcription factors are considered key factors in redox signaling because they control the transcription of ROS-scavenging enzymes and, inversely, FoxO activity is post-translationally modulated by ROS (de Keizer *et al.*, 2011). Recently, the cysteine residues in FoxO have been reported to act as sensors for the local redox state and to be involved in the interaction with nuclear import receptor transportin-1 via disulfide formation. The different redox states result in varying stability of the interaction and extent of FoxO nuclear translocation (Putker *et al.*, 2013). This finding may explain why antioxidant doses higher than 100 nM do not consistently enhance, but rather reduce, FoxO3a nuclear translocation. The reduced FoxO3a nuclear translocation at antioxidant concentrations higher than 100 nM may be partly because of cellular defense mechanisms against hyper-reductive conditions, for which cells activate a signaling pathway for reoxidation initiated by the FoxO protein after sensing a hyper-reductive state for a physiological balance between the oxidative and reductive states. High levels of antioxidants may induce a hyper-reductive state inside cells, which inhibits disulfide formation with binding partners, reduces FoxO nuclear translocation, and reduces the transcription of ROS-scavenging enzymes.

Because FoxO3a overexpression or activation using PI3K inhibitors influenced the expression of MITF, TYR, TYRP-1, and DCT (Figure 3), FoxO3a activation by antioxidants appears to affect selectively the expression of TYR and DCT (Figure 4), suggesting that antioxidants most likely influence the expression of melanogenesis-related genes through mechanisms other than the FoxO3a pathway. In addition, various dose ranges of antioxidant and PI3K inhibitors could not recover the increased melanin caused by pretreatment with FoxO3a siRNA (Figure 6), indicating that FoxO3a activation is a major factor for this phenomenon.

Possible interaction of FoxO3a with other melanogenic proteins

Although FoxO3a was proven to be a factor for the regulation of melanogenesis in our study, the mechanism by which FoxO3a regulates many melanogenesis-related genes remains unknown. Because FoxO3a interacts and forms a complex with many proteins, FoxO3a may bind to melanogenesis-related factors working within the nucleus to generate high-molecular-weight complexes and perform functions that are associated with melanogenesis. The FoxO family members contain one forkhead-binding domain fused with the transcriptional activation domain of the respective forkhead factor, which interacts with the DNA-binding domain of other transcription factors, thereby generating a chimeric form (Van der Vos *et al.*, 2011a, 2011b). Similarly, the PAX3-FoxO1 chimeric form has been found in alveolar rhabdomyosarcoma and consists of the transactivation and DNA-binding domains of FoxO1 and PAX3, respectively (Hu *et al.*, 2008), suggesting that PAX3 and FoxO3a may interact. Another putative binding target of FoxO3a is peroxisome

proliferator–activated receptor gamma coactivator-1α, which activates MITF in human melanoma cell lines and is regulated by alpha-melanocyte stimulating hormone (α-MSH) (Shoag *et al.*, 2013). Peroxisome proliferator–activated receptor gamma coactivator -1α is regulated by FoxO factors transcriptionally and post-translationally and can cooperate with FoxO (Corton and Brown-Borg, 2005). Considering that our results show that the deletion of FoxO3a using siRNA upregulated the PAX3 and MITF mRNA levels and that FoxO3a activation via PI3K inhibitors reversed this effect, PAX3 and peroxisome proliferator–activated receptor gamma coactivator-1α do not appear to be direct transcriptional targets of FoxO3a. However, these factors may still represent binding partners of FoxO3a for melanogenesis regulation. We also speculate that other factors such as SCF, cKIT, and SOX10, for which the expression levels correlated with FoxO3a expression and activation, may be transcriptional targets of FoxO3a.

In conclusion, inhibiting melanogenesis by manipulating the production of ROS or the activity of cellular regulators of ROS has been considered an attractive therapeutic target for hyperpigmentation and melanoma. Typically, pharmacological agents have been designed to reduce melanin synthesis through the antioxidative process and have focused primarily on eliminating ROS. On the basis of our study, FoxO3a mediates the antioxidant effect on cellular functions, including melanogenesis, and may represent a potent target for regulating those signaling pathways. Because FoxO3a is associated with longevity in humans and in *C. elegans*, which is a model organism for studying aging, this study suggests that FoxO3 may represent a potent target for pursuing both antiaging and antimelanogenic effects.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies against MITF (C5), TYR (H-109), TYRP-1 (H-90), DCT (C-9), GAPDH (FL-335), and lamin B (C-5) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MITF (Neomarkers, Fremont, CA) and TYR (Upstate Biotechnology, Lake Placid, NY) antibodies were also used. FoxO3a (no. 9467), FoxO3a (75D8), FoxO1 (C29H4), FoxO4 (55D4), phospho-FoxO3a (no. 9466), Akt (no. 9272), and phosphor-Akt (no. 4060) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The PI3K inhibitors LY294002 and wortmannin were purchased from Cell Signaling Technology. Akt inhibitor 1/2 (A6730) was obtained from Sigma (St Louis, MO). Secondary antibodies for western blot and immunofluorescence were obtained from Cell Signaling Technology and Invitrogen (Carlsbad, CA), respectively. Plasmids for HA-FoxO3a WT (1787) and the NLS mutant, FLAG-FoxO3a TM (8361), were obtained from Addgene (Cambridge, MA). FoxO3a-validated stealth RNA interference (RNAi) duopak and scramble RNAi were purchased from Invitrogen.

Cell culture and growth activity assay

MNT1 cells were maintained in minimum essential media (Gibco, Grand Island, NY) containing 10% DMEM, 20 mM HEPES (Sigma), 20% fetal bovine serum, 100 U ml^{-1} penicillin G, and 100 μg ml^{-1} streptomycin sulfate. MNT1 cells were incubated at 37 °C with 5% CO_2 and regularly passaged at a density of 80% (1:8 ratio). Cell proliferation and cytotoxicity were measured using cell counting kit-8 (Dojindo, Kumamoto, Japan).

TYR enzymatic activity assay and determination of melanin levels

To assess cellular TYR activity, equal amounts of cell lysates (10 μg) were incubated with 10 mM L-dihydroxyphenylalanine (pH 6.8) at 37 °C for 1 hour. Melanin synthesized from L-dihydroxyphenylalanine by TYR in the cell extracts was measured at 490 nm in a UV–vis spectrometer (Molecular Devices, Sunnyvale, CA). Mushroom TYR catalytic activity was measured by incubating 2 mg ml^{-1} mushroom TYR (Sigma) with 10 mM L-dihydroxyphenylalanine solution supplemented with the reagents of interest at 37 °C for 1 hour. Melanin levels were measured at 490 nm. To measure cellular melanin levels, the cell pellets were dissolved in 1 N sodium hydroxide, and melanin levels were determined by measuring absorbance at 490 nm. The melanin levels were normalized to protein input.

Small interfering RNA and plasmid transfection

MNT1 cells cultured in 60 mm dishes were transfected with stealth RNAi duopak FoxO3a (cat. no. 45-1712; Invitrogen), SignalSilence Akt siRNA (no. 6211; Cell Signaling Technology), and a Stealth RNAi siRNA negative control kit (cat. no. 12935-100; Invitrogen) using Lipofectamine RNAi MAX (Invitrogen) and 5 nM siRNA for 48 hours, according to the manufacturer's instructions. MNT1 cells (3.5×10^5 cells per 6 wells) were grown to 60–70% confluence and then transfected with 4 μg of plasmid. The plasmids included constitutively active HA-FoxO (1787; Addgene) and constitutively active FoxO3a NLS mutant, FLAG-FoxO3a TM (8367; Addgene).

FoxO localization assay using immunofluorescence

MNT1 cells were treated with each reagent (Vc, NAC, and Trolox) for 4 days, washed with phosphate-buffered saline, fixed for 30 minutes in 4% paraformaldehyde, washed again, and incubated for 10 min in 0.1% Triton X-100. The cells were washed three times in phosphate-buffered saline and incubated with anti-FoxO3a (1:200) or anti-FoxO1 antibodies (1:200) diluted in Hank's solution (0.44 mM KH_2PO_4, 5.37 mM KCl, 0.34 mM Na_2HPO_4, 136.89 mM NaCl, and 5.55 mM D-glucose) at 4 °C overnight. Secondary antibodies (Alexa Fluor 555– or Alexa Fluor 488–conjugated goat anti-rabbit or -mouse) were added for 1 hour at room temperature. After washing, the coverslips were mounted onto glass slides and visualized using an EVOSfl digital fluorescence microscope (Advanced Microscopy Group, Mill Creek, WI) (Ex: 555 nm, Em: 565 nm for FoxO3a; Ex: 488 nm, Em: 519 nm for FoxO1).

Cellular fractionation assay

Cytoplasmic and nuclear fractions were prepared according to a previously published protocol (Schreiber *et al.*, 1989). Briefly, the cells were lysed in a cytosolic lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 10% NP-40, 0.5 mM phenylmethyl sulfonyl, 1 mM dithiothretiol, and protease inhibitors. The nuclei were pelleted via a short centrifugation step and lysed in a nuclear buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethyl sulfonyl, 1 mM dithiothretiol, and protease inhibitors. The nuclear pellets were subjected to three freeze–thaw cycles before they were sonicated and centrifuged to obtain a solubilized nuclear fraction.

Western blot analysis

MNT1 cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothretiol, 0.5% NP-40, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM phenylmethyl sulfonyl, and a protease inhibitor cocktail (protease inhibitor; Sigma) and incubated with the appropriate antibodies for 1 hour. This incubation was followed by the addition of precleared protein G beads (GE Healthcare, Chalfont St Giles, UK) overnight at 4 °C. Next, the beads were washed five times with lysis buffer. Western blotting was performed following standard protocols. The cell lysates (20 μg protein) were boiled in SDS sample buffer and resolved using 4–12% SDS-PAGE. The proteins were then transferred to a polyvinylidene difluoride membrane (Invitrogen) and probed using specific antibodies.

Quantitative real-time reverse-transcriptase–PCR

MNT1 cells were homogenized in TRIzol reagent (Gibco), and total RNA was extracted according to a standard protocol. Total RNA (4 μg) was reverse-transcribed using random hexamers and Super-Script III (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using the ABI 7500 Fast Real-Time PCR System with Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA) and TaqMan site-specific primers and probes (Applied Biosystems). We used the $\Delta\Delta C_T$ analysis method (Schmittgen and Livak, 2008); the efficiency of the target and the reference amplification were approximately equal. Reactions were performed in triplicate, and mRNA expression levels were quantified using the relative C_T method and normalized to *GAPDH* level.

Statistical analysis

For quantitative real-time reverse-transcriptase–PCR (RT-qPCR), data are shown as the mean ± SD of at least three triplicate measurements. The *P*-values generated after a two-tailed Student's *t*-test were used to compare ΔC_T values between the control and treatment groups. All statistical tests were two-sided, and the threshold for statistical significance was 0.05 ($^*P<0.05$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The MNT1 cell line was kindly provided by Dr Ai-Young Lee (Dongguk University, Seoul, South Korea).

Author contributions

The authors have made the following declarations regarding their contributions. JK, HC, EGC, and TRL conceived and designed the experiments. JK and HC performed the experiments. JK, HC, EGC, and TRL analyzed the data. JK, HC, EGC, and TRL wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Bi W, Xiao L, Jia Y *et al.* (2010) c-Jun N-terminal kinase enhances MST1-mediated pro-apoptotic signaling through phosphorylation at serine 82. *J Biol Chem* 285:6259–64

Biggs WH, Cavenee WK, Arden KC (2001) Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. *Mamm Genome* 12:416–25

Blucher M, Kahn BB, Kahn CR (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* 299:572–4

Brunet A, Bonni A, Zigmond MJ *et al.* (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* 96:857–68

Brownawell AM, Kops GJ, Macara IG *et al.* (2001) Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Mol Cell Biol* 21: 3534–46

Burgering BM, Kops GJ (2002) Cell cycle and death control: long live forkhead. *Trends Biochem Sci* 27:352–60

Castrillon DH, Miao L, Kollipara R *et al.* (2003) Suppression of ovarian follicle activation in mice by the transcription factor FoxO3a. *Science* 301: 215–8

Cornelius C, Perrotta R, Graziano A *et al.* (2013) Stress responses, vitagenes and hormesis as critical determinants in aging and longevity: Mitochondria as a ''chi''. *Immun Ageing* 10:15

Corton JC, Brown-Borg HM (2005) Peroxisome proliferator-activated receptor gamma coactivator 1 in caloric restriction and other models of longevity. *J Gerontol A* 60:1494–509

de Keizer PL, Burgering BM, Dansen TB (2011) Forkhead box o as a sensor, mediator, and regulator of redox signaling. *Antioxid Redox Signal* 14:1093–106

Essers MA, Weijzen S, de Vries-Smits AM *et al.* (2004) FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 23:4802–12

Fujiwara Y, Sahashi Y, Aritro M *et al.* (2004) Effect of simultaneous administration of vitamin C, L-cysteine and vitamin E on the melanogenesis. *Biofactors* 21:415–8

Hayakawa R (1980) Clinical research group on a combination preparation of vitamin E and C. Effects of combination preparation of vitamin E and C in comparison with single preparation to the patients of facial hyperpigmentation: a double-blind controlled clinical trial. *Nishinihon J Dermatol* 42:1024–34

Heffetz D, Rutter WJ, Zick Y (1992) The insulinomimetic agents H_2O_2 and vanadate stimulate tyrosine phosphorylation of potential target proteins for the insulin receptor kinase in intact cells. *Biochem J* 288: 631–5

Holzenberger M, Dupont J, Ducos B *et al.* (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421:182–7

Hosaka T, Biggs WH, Tieu D *et al.* (2004) Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci USA* 101:2975–80

Hu MC, Lee DF, Xia W *et al.* (2004) IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* 117:225–37

Hu P, Geles KG, Paik JH *et al.* (2008) Codependent activators direct myoblast-specific MyoD transcription. *Dev Cell* 15:534–46

Hu ZM, Zhou Q, Lei TC *et al.* (2009) Effects of hydroquinone and its glucoside derivatives on melanogenesis and antioxidation: biosafety as skin whitening agents. *J Dermatol Sci* 55:179–84

Jacobs FM, Van der Heide LP, Wijchers PJ *et al.* (2003) FoxO6, anovel member of the FoxO class of transcription factors with distinct shuttling dynamics. *J Biol Chem* 278:35959–67

Kampkötter A, Gombitang Nkwonkam C, Zurawski RF *et al.* (2007) Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism *Caenorhabditis elegans*. *Arch Toxicol* 12:849–58

Kim HK, Kim YK, Song IH *et al.* (2005) Down-regulation of a forkhead transcription factor, FoxO3a, accelerates cellular senescence in human dermal fibroblasts. *J Gerontol* 60A:4–9

Kops GJ, Medema RH, Glassford J *et al.* (2002) Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 22:2025–36

Lerner AB, Fitzpatrick TB (1950) Biochemistry of melanin formation. *Physiol Rev* 30:91–126

Mandinova H, Lefort K, di Vignano AT *et al.* (2008) The FoxO3a gene is a key negative target of canonical Notch signaling in the keratinocyte UVB response. *EMBO J* 27:1243–54

Nakae J, Biggs WH, Kitamura T *et al.* (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* 3:3

Obsil T, Obsilova V (2008) Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene* 27:2263–75

Paik JH, Kollipara R, Chu G *et al.* (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* 128:309–23

Patridge L, Brüning JC (2008) Forkhead transcription factors and ageing. *Oncogene* 27:2351–63

Picardo M, Maresca V, Elibenschutz L *et al.* (1999) Correlation between antioxidants and phototypes in melanocyte cultures. A possible link of physiologic and pathologic relevance. *J Invest Dermatol* 113: 424–5

Putker M, Madl T, Vos HR *et al.* (2013) Redox-dependent control of FOXO/DAF-16 by transportin-1. *Mol Cell* 49:1–13

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C_T method. *Nat Protocols* 3:1101–8

Schreiber E, Matthias P, Muller MM *et al.* (1989) Rapid detection of octamer binding-proteins with mini-extracts, prepared from a small number of cells. *NucleicAcids Res* 17:6419

Shoag J, Haq R, Zhang M *et al.* (2013) PGC-1 coactivators regulate MITF and the tanning response. *Mol Cell* 49:1–13

Ueda S, Masutani H, Nakamura H *et al.* (2002) Redox control of cell death. *Antioxid Redox Signal* 4:405–14

Van der heide LP, Hoekman MFM, Smidt MP (2004) The ins and outs FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem J* 380:297–309

Van der Vos KE, Coffer PJ (2011a) FoxO-binding partners: it takes two to tango. 2008 *Oncogene* 27:2289–99

Van der Vos KE, Coffer PJ (2011b) The extending network of FOXO transcriptional target genes. *Antioxid Redox Signal* 14:579–92

Wang X, Chen WR, Xing D (2012) A pathway from JNK through decreased ERK and Akt activities for FoxO3a nuclear translocation in response to UV irradiation. *J Cell Physiol* 227:1168–78

Yao R, Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 267:2003–6